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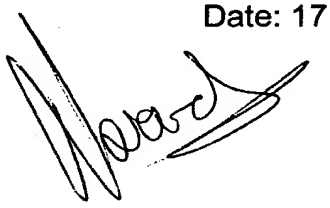

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IMPROVED METHOD FOR TRANSFERRING NUCLEIC ACID INTO THE STRIATED MUSCLE AND COMBINATION TO EFFECT THE PROCESS

The present invention relates to a very remarkable improvement in the in vivo transfer into striated muscle cells of nucleic acids or of nucleic acids combined with products which make it possible to increase the yield of such transfers, and to the combination of a nucleic acid and the method of transfer according to the invention for their use for gene therapy.

The transfer of genes into a given cell is at the root of gene therapy. However, one of the problems is to succeed in causing a sufficient quantity of nucleic acid to penetrate into cells of the host to be treated; indeed, this nucleic acid, in general a gene of interest, has to be expressed in transfected cells. One of the approaches selected in this regard has been the integration of the nucleic acid into viral vectors, in particular into retroviruses, adenoviruses or adenoassociated viruses. These systems take advantage of the cell penetration mechanisms developed by viruses, as well as their protection against degradation. However, this approach has disadvantages, and in particular a risk of production of infectious viral particles capable of dissemination in the host organism, and, in the case of retroviral vectors, a risk of insertional mutagenesis. Furthermore, the capacity for insertion of a therapeutic or vaccinal gene into a viral genome remains limited.

In any case, the development of viral vectors capable of being used in gene therapy requires the use of complex techniques for defective viruses and for complementation cell lines.

Another approach (Wolf et al. Science 247, 1465-68, 1990; Davis et al. Proc. Natl. Acad. Sci. USA 93, 7213-18, 1996) has therefore consisted in administering into the muscle or into the blood stream a nucleic acid of a plasmid nature, combined or otherwise with compounds intended to promote its

transfection, such as proteins, liposomes, charged lipids or cationic polymers such as polyethylenimine, which are good transfection agents in vitro (Behr et al. Proc. Natl. Acad. Sci. USA 86, 6982-6, 1989; Felgner et al. Proc. Natl. Acad. Sci. USA 84, 7413-7, 1987; Boussif et al. Proc. Natl. Acad. Sci. USA 92, 7297-301, 1995).

Since the initial publication by J. A. Wolff et al. showing the capacity of muscle tissue to incorporate DNA injected in free plasmid form (Wolff et al. Science 247, 1465-1468, 1990), numerous authors have tried to improve this procedure (Manthorpe et al., 1993, Human Gene Ther. 4, 419-431; Wolff et al., 1991, BioTechniques 11, 474-485). A few trends emerge from these tests, such as in particular:

- the use of mechanical solutions to force the entry of DNA into cells by adsorbing the DNA onto beads which are then propelled onto the tissues ("gene gun") (Sanders Williams et al., 1991, Proc. Natl. Acad. Sci. USA 88, 2726-2730; Fynan et al., 1993, BioTechniques 11, 474-485). These methods have proved effective in vaccination strategy but they affect only the top layers of the tissues. In the case of the muscle, their use would require a surgical approach in order to allow access to the muscle because the particles do not cross the skin tissues;

- the injection of DNA, no longer in free plasmid form but combined with molecules capable of serving as vehicle facilitating the entry of the complexes into cells. Cationic lipids, which are used in numerous other transfection methods, have proved up until now disappointing as regards application into the muscle tissue, because those which have been tested have been found to inhibit transfection (Schwartz et al., 1996, Gene Ther. 3, 405-411). Likewise for cationic peptides and polymers (Manthorpe et al., 1993, Human Gene Ther. 4, 419-431). The only case of a favourable combination appears to be the mixing of poly(vinyl alcohol) or polyvinylpyrrolidone with DNA. The increase resulting from these combinations only represents a factor

of less than 10 compared with DNA injected in naked form (Mumper et al., 1996, Pharmaceutical Research 13, 701-709);

- the pretreatment of the muscle to be injected with solutions intended to improve the diffusion and/or the stability of DNA (Davis et al., 1993, Hum. Gene Ther. 4, 151-159), or to promote the entry of nucleic acids, for example the induction of cell multiplication or regeneration phenomena. The treatments have involved in particular the use of local anaesthetics or of cardiotoxin, of vasoconstrictors, of endotoxin or of other molecules (Manthorpe et al., 1993, Human Gene Ther. 4, 419-431; Danko et al., 1994, Gene Ther. 1, 114-121; Vitadello et al., 1994, Hum. Gene Ther. 5, 11-18). These pretreatment protocols are difficult to manage, bupivacaine in particular requiring, in order to be effective, being injected at doses very close to lethal doses. The preinjection of hyperosmotic sucrose, intended to improve diffusion, does not increase the transfection level in the muscle (Davis et al., 1993).

Electroporation, or use of electric fields to permeabilize cells, is also used in vitro to promote the transfection of DNA into cells in culture. However, it has up until now been accepted that this phenomenon responded to an effect which is dependent on a threshold and that this electropermeabilization could only be observed for electric fields of relatively high intensity, of the order of 800 to 1200 volts/cm for animal cells. This technique has also been proposed in vivo to improve the efficacy of antitumour agents, such as bleomycin, in solid tumours in man (American U.S. Pat. No. 5,468,228, L. M. Mir). With pulses of very short duration (100 microseconds), these electrical conditions (800 to 1200 volts/cm) are very well suited to the intracellular transfer of small molecules. These conditions (pulses of 100 microseconds) have been applied with no improvement for the transfer of nucleic acids in vivo into the liver, where fields of less than 1000 volts/cm have proved completely ineffective, and even inhibitory compared

with the injection of DNA in the absence of electrical impulses (Patent WO 97/07826 and Heller et al. FEBS Letters, 389, 225-8, 1996).

There are in fact difficulties with applying this technique in vivo because the administration of fields of such an intensity may cause extensive tissue lesions to a greater or lesser extent which do not represent a problem for the treatment of cancer patients but which may have a major disadvantage for the healthy subject or the sick subject when the nucleic acid is administered into tissues other than tumour tissues, in particular into the striated muscle.

Whereas all the studies cited mention the need for high electric fields, of the order of 1000 volts/cm, to be effective in vivo, in a truly unexpected and remarkable manner, the applicants have now shown that the transfer of nucleic acids into muscles in vivo could be very substantially increased, without undesirable effects, by subjecting the muscle to electrical pulses of low intensity, for example 100 or 200 volts/cm and of a relatively long duration. Furthermore, the applicants have observed that the high variability in the expression of the transgene observed in the prior art for the transfer of DNA into the muscle was notably reduced by the method according to the invention.

Accordingly, the present invention relates to a method of transferring nucleic acids into one or more striated muscles in vivo, in which the muscle cells are brought into contact with the nucleic acid to be transferred, by direct administration into the tissue or by topical or systemic administration, and in which the transfer is brought about by application to the said muscles of one or more electrical pulses of an intensity between 1 and 800 volts/cm.

Preferably, the intensity of the field is between 4 and 400 volts/cm and the total duration of application is greater than 10 milliseconds. The number of pulses used is, for example, from 1 to 100,000 pulses and the frequency of the pulses is between 0.1 and 10,000 Hertz. Preferably, the frequency of the pulses is between 0.2 and 100 Hertz. The pulses may also be delivered in an irregular manner and the function which describes the intensity of the field as a function of time may be

variable. The integral of the function describing the variation of the electric field with time is greater than 1 kV.times.msec/cm. According to a preferred mode of the invention, this integral is greater than or equal to 5 kV.times.msec/cm.

According to a preferred mode of the invention, the field intensity of the pulses is between 30 and 300 volts/cm.

The electrical pulses are chosen from square wave pulses, electric fields generating exponentially decreasing waves, oscillating unipolar waves of limited duration, oscillating bipolar waves of limited duration, or other wave forms. According to a preferred mode of the invention, the electrical pulses are square wave pulses.

The administration of electrical pulses may be carried out by any method known to persons skilled in the art, for example:

- system of external electrodes placed on either side of the tissue to be treated, in particular non-invasive electrodes placed in contact with the skin,
- system of electrodes implanted in the tissues,
- system of electrodes/injector allowing the simultaneous administration of the nucleic acids and the electric field.

The administration being carried out in vivo, it is sometimes necessary to use intermediate products which provide electrical continuity with the non-invasive external electrodes. This may be for example an electrolyte in gel form.

The nucleic acids may be administered by any appropriate means, but are preferably injected in vivo directly into the muscle or administered by another route, local or systemic, which makes them available at the site of application of the electric field. The nucleic acids may be administered with agents allowing or facilitating transfer, as was mentioned above. In particular, these nucleic acids may be free in solution or combined with synthetic agents, or carried by viral vectors. The synthetic agents may be lipids or polymers known to a person skilled in the art, or alternatively targeting elements allowing attachment to the

membrane of the target tissues. Among these elements, there may be mentioned vectors carrying sugars, peptides, antibodies or hormone receptors.

It can be understood, under these conditions of the invention, that the administration of the nucleic acids can preceded, be simultaneous with or even subsequent to the application of the electric fields.

Accordingly, the subject of the present invention is also a nucleic acid and an electric field of an intensity between 1 and 800 volts/cm, as combination product for their administration simultaneously, separately or spaced out over time, to the striated muscle in vivo. Preferably, the intensity of the field is between 4 and 400 volts/cm and, more preferably still, the intensity of the field is between 30 and 300 volts/cm.

The method according to the present invention can be used in gene therapy, that is to say therapy in which the expression of a transferred gene, but also the modulation or the blocking of a gene, makes it possible to provide the treatment of a particular pathological condition.

Preferably, the muscle cells are treated for the purpose of a gene therapy allowing:

- either the correction of dysfunctions of the muscle cells themselves (for example for the treatment of myopathies linked to genetic deficiencies),
- or the safeguard and/or the regeneration of the vascularization or the innervation of the muscle or of other muscles or organs by trophic, neurotrophic and angiogenic factors produced by the transgene,
- or the transformation of the muscle into an organ secreting products leading to a therapeutic effect such as the product of the gene itself (for example factors for regulation of thrombosis and of haemostasis, trophic factors, hormones such as insulin and the like) or such as an active metabolite synthesized in the muscle by virtue of the addition of the therapeutic gene,
- or a vaccine or immunostimulant application.

Another subject of the invention is the combination of the electrical pulses of a field with compositions containing nucleic acids formulated for any administration allowing access to a striated muscle by the topical, cutaneous, oral, vaginal, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular or transdermal route, and the like. Preferably, the pharmaceutical compositions of the invention contain a pharmaceutically acceptable vehicle for an injectable formulation, in particular for a direct injection into the desired organ, or for any other administration. They may be in particular isotonic sterile solutions or dry, in particular freeze-dried, compositions which, upon addition, depending on the case, of sterilized water or of physiological saline, allow the preparation of injectable solutions. The nucleic acid doses used for the injection as well as the number of administrations and the volume of injections may be adjusted according to various parameters, and in particular according to the mode of administration used, the relevant pathological condition, the gene to be expressed, or the desired duration of treatment.

The nucleic acids may be of synthetic or biosynthetic origin, or may be extracted from viruses or prokaryotic cells or from eukaryotic cells derived from unicellular organisms (for example yeasts) or from pluricellular organisms. They may be administered in combination with all or part of the components of the organism of origin and/or of the synthesis system.

The nucleic acid may be a deoxyribonucleic acid or a ribonucleic acid. It may be sequences of natural or artificial origin, and in particular genomic DNA, cDNA, mRNA, tRNA and rRNA, hybrid sequences or synthetic or semisynthetic sequences of modified or unmodified oligonucleotides. These nucleic acids may be obtained by any technique known to persons skilled in the art, and in particular by targeting libraries, by chemical synthesis or by mixed methods including chemical or enzymatic modification of sequences obtained by targeting libraries. They may be chemically modified.

In particular, the nucleic acid may be a DNA or a sense or antisense RNA or an RNA having catalytic property such as a ribozyme. "Antisense" is understood to mean a nucleic acid having a sequence complementary to a target sequence, for example an mRNA sequence the blocking of whose expression is sought by hybridization with the target sequence. "Sense" is understood to mean a nucleic acid having a sequence which is homologous or identical to a target sequence, for example a sequence which binds to a protein transcription factor and which is involved with the expression of a given gene. According to a preferred embodiment, the nucleic acid comprises a gene of interest and elements allowing the expression of the said gene of interest. Advantageously, the nucleic acid fragment is in the form of a plasmid.

The deoxyribonucleic acids may be single- or double-stranded, as well as short oligonucleotides or longer sequences. They may carry therapeutic genes, sequences for regulation of transcription or of replication, or regions for binding to other cellular components, and the like. For the purposes of the invention, "therapeutic gene" is understood to mean in particular any gene encoding an RNA or a protein product having a therapeutic effect. The protein product encoded may be a protein, a peptide, etc. This protein product may be homologous in relation to the target cell (that is to say a product which is normally expressed in the target cell when the latter exhibits no pathological condition). In this case, the expression of the transgene makes it possible, for example, to overcome an insufficient expression in the cell or the expression of an inactive or weakly active protein due to a modification, or makes it possible to overexpress the said protein. The therapeutic gene may also encode a mutant of a cellular protein having increased stability or a modified activity, etc. The protein product may also be heterologous in relation to the target cell. In this case, an expressed protein may, for example, supplement or provide an activity which is deficient in the cell (treatment of myopathies or of enzymatic deficiencies), or may make it possible to combat a pathological condition, or to stimulate an immune response.

Among the therapeutic products for the purposes of the present invention, there may be mentioned more particularly enzymes, blood derivatives, hormones, such as insulin, or growth hormone, lymphokines: interleukins, interferons, TNF, etc. (French Patent No. 92 03120), growth factors, for example angiogenic factors such as VEGF or FGF, neurotransmitters or their precursors or synthesis enzymes, trophic factors, in particular neurotrophic factors for the treatment of neurodegenerative diseases, of traumas which have damaged the nervous system, or of retinal degeneration; BDNF, CNTF, NGF, IGF, GMF, aFGF, NT3, NT5, HARP/pleiotrophin, or bone growth factors, haematopoietic factors etc., dystrophin or a minidystrophin (French Patent No. 91 11947), genes encoding factors involved in coagulation: Factors VII, VIII, IX, suicide genes (thymidine kinase, cytosine deaminase), genes for haemoglobin or other protein transporters, genes corresponding to the proteins involved in the metabolism of lipids, of the apolipoprotein type chosen from apolipoproteins A-I, A-II, A-IV, B, C-I, C-II, C-III, D, E, F, G, H, J and apo(a), metabolic enzymes such as for example lipoprotein lipase, hepatic lipase, lecithin-cholesterol acyltransferase, 7-alpha-cholesterol hydroxylase, phosphatidyl acid phosphatase, or lipid transfer proteins such as the cholesterol ester transfer protein and the phospholipid transfer protein, an HDL-binding protein or a receptor chosen for example from the LDL receptors, the remnant chylomicron receptors and the scavenger receptors, etc. Leptin may furthermore be added to treat obesity.

Among the other proteins or peptides which may be secreted by the muscle, it is important to underline the antibodies, the variable fragments of single-chain antibody (ScFv) or any other antibody fragment possessing recognition capacities for its use in immunotherapy, for example for the treatment of infectious diseases, of tumours, of autoimmune diseases such as multiple sclerosis (antiidiotype antibodies). Other proteins of interest are, in a nonlimiting manner, soluble receptors such as, for example, the soluble CD4 receptor or the soluble receptor for TNF for anti-HIV therapy, the soluble receptor for

acetylcholine for the treatment of myasthenia; substrate peptides or enzyme inhibitors, or peptides which are agonists or antagonists of receptors or of adhesion proteins such as, for example, for the treatment of asthma, thrombosis, restenosis; artificial, chimeric or truncated proteins. Among the hormones of essential interest, there may be mentioned insulin in the case of diabetes, growth hormone and calcitonin.

The numerous examples which precede and those which follow illustrate the potential scope of the field of application of the present invention.

The therapeutic nucleic acid may also be an antisense sequence or gene, whose expression in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs. Such sequences may, for example, be transcribed in the target cell into RNA complementary to cellular mRNAs and thus block their translation into protein, according to the technique described in European Patent No. 140 308. The therapeutic genes also comprise the sequences encoding ribozymes, which are capable of selectively destroying target RNAs (European Patent No. 321 201).

As indicated above, the nucleic acid may also comprise one or more genes encoding an antigenic peptide capable of generating an immune response in humans or in animals. In this particular embodiment, the invention therefore allows either the production of vaccines, or the carrying out of immunotherapeutic treatments applied to humans or to animals, in particular against microorganisms, viruses or cancers. It may be in particular antigenic peptides specific for the Epstein-Barr virus, the HIV virus, the hepatitis B virus (European Patent No. 185 573), the pseudo-rabies virus, the "syncytia forming virus", other viruses or antigens specific for tumours such as the MAGE proteins (European Patent No. 259 212).

Preferably, the nucleic acid also comprises sequences allowing and/or promoting the expression, in the muscle, of the therapeutic gene and/or of the gene encoding the antigenic peptide. They may be sequences which are naturally

responsible for the expression of the gene considered when these sequences are capable of functioning in the transfected cell. They may also be sequences of different origin (responsible for the expression of other proteins, or even synthetic). In particular, they may be promoter sequences of eukaryotic or viral genes. For example, they may be promoter sequences derived from the genome of the cell which it is desired to transfect. Likewise, they may be promoter sequences derived from the genome of a virus. In this regard, there may be mentioned the promoters of the EIA, MLP, CMV, RSV genes, etc. In addition, these expression sequences may be modified by the addition of activating or regulatory sequences. It may be a promoter, inducible or repressible.

Moreover, the nucleic acid may also comprise, in particular upstream of the therapeutic gene, a signal sequence directing the therapeutic product synthesized in the secretory pathways of the target cell. This signal sequence may be the natural signal sequence of the therapeutic product, but it may also be any other functional signal sequence, or an artificial signal sequence. The nucleic acid may also comprise a signal sequence directing the synthesized therapeutic product towards a particular compartment of the cell.

Other genes which are of interest have been described in particular by McKusick, V. A. Mendelian (Inheritance in man, catalogs of autosomal dominant, autosomal recessive, and X-linked phenotypes. Eighth edition, John Hopkins University Press (1988)), and in Stanbury, J. B. et al. (The metabolic basis of inherited disease, Fifth edition, McGraw-Hill (1983)). The genes of interest cover the proteins involved in the metabolism of amino acids, lipids and other constituents of the cell.

There may thus be mentioned, with no limitation being implied, the genes associated with diseases of carbohydrate metabolism such as for example fructose-1-phosphate aldolase, fructose-1,6-diphosphatase, glucose-6-phosphatase, lysosomal α-1,4-glucosidase, amylo-1,6-glucosidase, amylo-(1,4:1,6)-transglucosidase, muscle phosphorylase, muscle phosphofructokinase,

phosphorylase-β-kinase, galactose-1-phosphate uridyl transferase, all the enzymes of the pyruvate dehydrogenase complex, pyruvate carboxylase, 2-oxoglutarate glyoxylase carboxylase, D-glycerate dehydrogenase.

There may also be mentioned:

- the genes associated with diseases of amino acid metabolism such as for example phenylalanine hydroxylase, dihydrobiopterin synthetase, tyrosine aminotransferase, tyrosinase, histidinase, fumarylacetoacetase, glutathione synthetase, γ-glutamylcysteine synthetase, ornithine-δ-amino-transferase, carbamoylphosphate synthetase, ornithine carbamoyltransferase, argininosuccinate synthetase, argininosuccinate lyase, arginase, L-lysine dehydrogenase, L-lysine ketoglutarate reductase, valine transaminase, leucine isoleucine transaminase, decarboxylase for the branched-chain 2-keto acids, isovaleryl-CoA dehydrogenase, acyl-CoA dehydrogenase, 3-hydroxy-3-methylglutaryl-CoA lyase, acetoacetyl-CoA 3-ketothiolase, propionyl-CoA carboxylase, methylmalonyl-CoA mutase, ATP:cobalamine adenosyltransferase, dihydrofolate reductase, methylenetetrahydrofolate reductase, cystathionine β-synthetase, the sarcosine dehydrogenase complex, proteins belonging to the system for cleaving glycine, β-alanine transaminase, serum carnosinase, cerebral homocarnosinase;
- the genes associated with diseases of fat and fatty acid metabolism, such as for example lipoprotein lipase, apolipoprotein C-II, apolipoprotein E, other apolipoproteins, lecithin-cholesterol acyltransferase, LDL receptor, liver sterol hydroxylase, "phytanic acid" α-hydroxylase;
- the genes associated with lysosomal deficiencies, such as for example lysosomal α-L-iduronidase, lysosomal iduronate sulphatase, lysosomal heparan N-sulphatase, lysosomal N-acetayl-α-D-glucosaminidase, lysosomal acetyl-CoA:α-glucosamine N-acetyltransferase, lysosomal N-acetyl-α-D-glucosamine 6-sulphatase, lysosomal galactosamine 6-sulphate

sulphatase, lysosomal β-galactosidase, lysosomal arylsulphatase B, lysosomal β-glucuronidase, N-acetylglucosaminyl-phosphotransferase, lysosomal α-D-mannosidase, lysosomal α-neuraminidase, lysosomal aspartylglycosaminidase, lysosomal α-L-fucosidase, lysosomal acid lipase, lysosomal acid ceramidase, lysosomal sphingomyelinase, lysosomal glucocerebrosidase and lysosomal galactocerebrosidase, lysosomal galactosylceramidase, lysosomal arylsulphatase A, α.-galactosidase A, lysosomal acid β-galactosidase, α chain of lysosomal hexoamimidase A.

There may also be mentioned, in a nonrestrictive manner, the genes associated with diseases of steroid and lipid metabolism, the genes associated with diseases of purine and pyrimidine metabolism, the genes associated with diseases of porphyrin and haem metabolism, the genes associated with diseases of connective tissue, muscle and bone metabolism as well as the genes associated with blood diseases and diseases of the haematopoietic organs, muscle diseases (myopathy), diseases of the nervous system (neurodegenerative diseases) or diseases of the circulatory apparatus (treatment of ischaemias and of stenosis for example.

In the method according to the invention, the nucleic acid may be combined with any type of vector or any combination of these vectors which make it possible to improve the transfer of genes, for example, in a non limiting manner, with vectors such as viruses, synthetic or biosynthetic agents (for example lipidic, polypeptidic, glycosidic or polymeric agents), or beads which are propelled or otherwise. The nucleic acids may also be injected into a muscle which has been subjected to a treatment intended to improve the transfer of genes, for example a treatment of a pharmacological nature by local or systemic application, or an enzymatic, permeabilizing (use of surfactants), surgical, mechanical, thermal or physical treatment.

The advantage of the use of the muscle in gene therapy lies in numerous factors:

- the remarkable stability of the expression of the transgenes, more than several months, and therefore allowing the stable and sustained production of an intramuscular or secreted therapeutic protein,
- the ease of access to the muscle tissue, allowing a direct, rapid and non-dangerous administration into a non-vital organ,
- the large volume of the muscle mass, allowing multiple sites of administration,
- widely demonstrated secretory capacity of the muscle.

To these advantages, there may be added the safety provided by the local treatment linked to the use of local and targeted electric fields.

By virtue of all these advantages and the safety linked to the use of weak fields, the present invention could be applied in the region of the cardiac muscle for the treatment of cardiopathies, for example using a suitable defibrillator.

The combination of fields which are not very intense and which are administered over a long period, applied in particular to the muscles in vivo, improves the transfection of nucleic acids without causing notable damage to the tissues. These results improve the yield of DNA transfers within the context of gene therapy using nucleic acids.

Consequently, the advantages of the muscle tissue combined with the method according to the invention make it possible, for the first time, to envisage producing, by gene therapy, an agent at physiological and/or therapeutic doses, either in the muscle cells, or secreted in their vicinity or into the blood stream or the lymph circulation. Furthermore, the method according to the invention allows, for the first time, fine modulation and control of the effective quantity of transgene expressed by the possibility of modulating the volume of muscle tissue to be transfected, for example with multiple sites of administration, or the possibility of modulating the number, the shape, the surface and the arrangement

of the electrodes. An additional element of control comes from the possibility of modulating the efficiency of transfection by varying the field intensity, the number, the duration and the frequency of the pulses, and obviously according to the state of the art, the quantity and the volume of nucleic acids to be administered. It is thus possible to obtain an appropriate transfection level at the desired production or secretion level. The method finally allows increased safety compared with the chemical or viral methods for transferring genes in vivo, for which the affecting of organs other than the target organ cannot be completely excluded or controlled. Indeed, the method according to the invention allows control of the localization of the transfected tissues (strictly linked to the volume of tissue subjected to the local electrical pulses) and therefore provides the possibility of a return to the initial situation by complete or partial removal of the muscle, which is made possible by the non-vital character of this tissue and by its regeneration capacities. This great flexibility of use makes it possible to optimize the method according to the animal species (human and veterinary applications), the age of the subject, his physiological and/or pathological condition.

The method according to the invention makes it possible, in addition, for the first time, to transfect nucleic acids of large size unlike the viral methods which are limited by the size of the capsid. This possibility is essential for the transfer of genes of a very large size such as that for dystrophin or genes with introns and/or regulatory elements of large size, which is necessary for example for a physiologically regulated production of hormones. This possibility is essential for the transfer of episomes or of yeast artificial chromosomes or of minichromosomes.

The following examples are intended to illustrate the invention in a non limiting manner.

In these examples, reference will be made to the following figures:

- FIG. 1: effects of electrical pulses of high field intensity on the transfection of plasmid DNA pxl 2774 into the cranial tibial muscle in mice; mean values ± SEM,
- FIG. 2: effects of electrical pulses of intermediate field intensity on the transfection of plasmid DNA pxl 2774 into the cranial tibial muscle in mice; mean values ± SEM,
- FIG. 3: effects of electrical pulses of low field intensity and of different durations on the transfection of plasmid DNA pxl 2774 into the cranial tibial muscle in mice; mean values ± SEM,
- FIG. 4: effects of electrical pulses of low field intensity and of different durations on the transfection of plasmid DNA pxl 2774 into the cranial tibial muscle in mice; mean values ± SEM,
- FIG. 5: efficiency of electrotransfection of plasmid DNA pxl 2774 into the cranial tibial muscle of mice at low electric field intensities: mean values ± SEM.

EXAMPLE 1

Experiment Carried Out Under the Conditions of the Prior State of the Art in Which the Electric Fields Prove to be Inhibitors of Transfection

In this example, the following products were used:

DNA pxl 2774 (Patent PCT/FR 96/01414) is a plasmid DNA comprising the reporter gene for luciferase. Other products are available from commercial suppliers: Ketamine, Xylazine, physiological saline (0.9% NaCl).

An oscilloscope and a commercial generator of (rectangular or square) electrical pulses (electro-pulsator PS 15, Jouan, France) were used. The electrodes used are flat stainless steel electrodes distant from 5.3 mm.

The experiment is carried out on the mouse C57 B1/6. Mice from different cages are randomly separated before the experiment ("randomization").

The mice are anaesthetized with a ketamine and xylazine mixture. The plasmid solution (30 µl of a solution at 500 µg/ml of 0.9% NaCl) is injected longitudinally through the skin into the cranial tibial muscle of the left and right legs with the aid of a Hamilton syringe. The two electrodes are coated with a conducting gel and the injected leg is placed between the electrodes in contact with them.

The electrical pulses are applied perpendicularly to the axis of the muscle with the aid of a generator of square pulses one minute after the injection. An oscilloscope makes it possible to control the intensity in volts (the values indicated in the examples represent the maximal values), the duration in milliseconds and the frequency in hertz of the pulses delivered, which is 1 Hz. 8 consecutive pulses are delivered.

To evaluate the transfection of the muscle, the mice are humanely killed 7 days after the administration of the plasmid. The cranial tibial muscles of the left and right legs are then removed, weighed, placed in lysis buffer and ground. The suspension obtained is centrifuged in order to obtain a clear supernatant. The measurement of the luciferase activity is carried out on 10 µl of supernatant with the aid of a commercial luminometer in which the substrate is added automatically to the solution. The intensity of the luminescent reaction is given in RLU (Relative Luminescence Unit) for a muscle knowing the total volume of suspension. Each experimental condition is tested on 10 points: 5 animals injected bilaterally. Statistical comparisons are carried out with the aid of non-parametric tests. Two figures, of which the scale is linear or logarithmic, illustrate the results.

In this first experiment, the effects of an electric field of 800 to 1200 volts/cm which allows electroporation of tumours (Mir et al. Eur. J. Cancer 27, 68, 1991) were tested.

It is observed, according to FIG. 1, that relative to the control group, where the DNA is injected without an electrical pulse:

- with 8 pulses of 1200 volts/cm and of a duration of 0.1 msec, the mean value of the luciferase activity is much lower,
- with pulses of 1200 volts/cm and of 1 msec, 3 animals are dead, the mean value of the luciferase activity is much lower,
- with pulses of 800 volts/cm and of 1 msec, the mean value of the luciferase activity is also significantly reduced.

Most of the muscles which were subjected to the action of the electric field are visibly impaired (friable and of a whitish appearance).

EXAMPLE 2

Experiment for Electrotransfer of Nucleic Acids at Moderate Electric Fields

This experiment is carried out with C57 B1/6 mice. Apart from the electric field intensity of the pulses and their duration, the practical conditions are those of Example 1.

The results are shown in FIG. 2. The result of Example 1 is reproduced, that is to say the inhibitory effect of a series of 8 pulses at 800 volts/cm of a duration of 1 msec on the luciferase activity detected in the muscle. With a field of 600 volts/cm, the same inhibition and the same impairment of the muscle tissue are observed. On the other hand, in a remarkable and surprising manner, the decrease in voltage makes it possible to no longer visibly impair the muscles and, furthermore, at 400 and 200 volts/cm, the level of transfection of the muscles is on average greater than that obtained on the muscles not subjected to a field. It should be noted that, relative to the control group (not subjected to an electric field), the dispersion of the luciferase activity values is reduced at 200 volts/cm

(SEM=20.59% of the mean value against 43.32% in the absence of electric field (FIG. 2A)).

EXAMPLE 3

Experiment for Electrotransfer of Nucleic Acids with Pulses of Low Field Intensity Showing a Very High Stimulation of the Expression of the Transgene

This experiment is carried out with C57 B1/6 mice. Apart from the electric field intensity of the pulses and their duration, and the fact that the pulses are delivered 25 seconds after the injection of the DNA, the practical conditions are those of the preceding examples.

The results are shown in FIG. 3. The mean value of the expression of the luciferase transgene is markedly increased with a pulse duration of 20 msec at 100 volts/cm, and from a pulse duration of 5 msec at 200 volts/cm.

This experiment also clearly shows that the mean value of the luciferase activity obtained by electrotransfection of the DNA into the muscle is a function of the duration of the electrical pulses, when voltages of 200 and 100 volts/cm are used. It is also observed that the dispersion of the values is notably reduced for the electrotransfected muscle groups (FIG. 3A). In the absence of electrical pulses (control), the SEM represents 77.43% of the mean value whereas the relative SEM of the mean is reduced to 14% (200 volts/cm, 5 msec), 41.27% (200 volts/cm, 20 msec) and between 30% and 48% for the electrotransfer at 100 volts/cm of electric field.

Under the best condition for this experiment, the expression of the transgene is improved by a factor of 89.7 compared with the control injected in the absence of electrical pulses.

EXAMPLE 4

Experiment for Electrotransfer of Nucleic Acids into the Muscle at 200 Volts/cm Showing an Increase in the Expression of the Transgene by a Factor Greater than 200

This experiment is carried out in DBA 2 mice, with electrical pulses of a field intensity of 200 volts/cm and of variable duration, the other conditions of this experiment being those of Example 3.

This example confirms that at 200 volts/cm, the transfection of the luciferase activity is increased from a pulse duration of 5 msec and then continues to increase for longer durations (FIGS. 4 and 5). Here again, a reduction in the inter-individual variability indicated by the SEM relative to the non-electrotransfected control (the relative value of the SEM is equal to 35% for the control and 25, 22, 16, 18, 16 and 26% for series of pulses of 1, 5, 10, 15, 20 and 24 msec respectively), is observed with electrotransfection.

Under the best condition for this experiment, the expression of the transgene is improved by a factor of 205 relative to the control injected in the absence of electrical pulses.

EXAMPLE 5

Efficiency of the Electrotransfer of Nucleic Acids as a Function of the Product "Number of Pulses x Field Intensity x Duration of Each Pulse"

FIG. 5 exemplifies the importance of the parameter corresponding to the product "number of pulses x field intensity x duration of each pulse". This parameter in fact corresponds to the integral, as a function of time, of the function which describes the variation of the electric field.

The representation in FIG. 5 of the results obtained during experiments 2, 3 and 4 with electric field intensities of 200 V/cm, 100 V/cm or in the absence of electric fields shows that the transfection efficiency increases as a function of the product of the total duration of exposure to the electric field by the field intensity. A stimulating effect is obtained for a value greater than 1 kVxmsec/cm of the product "electric field x total duration of the pulses". According to a preferred mode, a stimulation is obtained for a value greater than or equal to 5 kV x msec/cm of the product "electric field x total duration of the pulses".

Claims

1. Method of transferring nucleic acid into one or more striated muscles in vivo in which the muscle cells are brought into contact with the nucleic acid to be transferred by direct administration into the tissue or by topical or systemic administration and in which the transfer is brought about by application to the said muscle of one or more electrical pulses of an intensity of between 1 and 800 volts/cm.

2. Method according to claim 1, characterized in that the intensity of the field is between 4 and 400 volts/cm.

3. Method according to claim 1, characterized in that the intensity of the field is between 30 and 300 volts/cm.

4. Method according to one of claims 1 to 3, characterized in that the total duration of application of the electric field is greater than 10 milliseconds.

5. Method according to one of claims 1 to 4, characterized in that the application, to the muscle, of the electric field comprises one or more pulses of regular frequency.

6. Method according to claim 5, characterized in that the application, to the muscle, of the electric field comprises between 1 and 100,000 pulses of frequency between 0.1 and 10,000 hertz.

7. Method according to one of claims 1 to 4, characterized in that the electrical pulses are delivered in an irregular manner relative to each other and in that the function describing the intensity of the electric field as a function of time for one pulse is variable.

8. Method according to one of claims 1 to 7, characterized in that the integral of the function describing the variation of the electric field with time is greater than 1 kVxmsec/cm.

9. Method according to claim 8, characterized in that this integral is greater than or equal to 5 kVxmsec/cm.

10. Method according to one of claims 1 to 9, characterized in that the electrical pulses are chosen from square wave pulses, electric fields generating exponentially decreasing waves, oscillating unipolar waves of limited duration, oscillating bipolar waves of limited duration, or other wave forms.

11. Method according to one of claims 1 to 10, characterized in that the electrical pulses comprise square wave pulses.

12. Method according to one of claims 1 to 11, characterized in that the electrical pulses are applied in an external manner.

13. Method according to one of claims 1 to 11, characterized in that the electrical pulses are applied inside the muscle.

14. Method according to one of claims 1 to 13, characterized in that the nucleic acid is injected into the muscle.

15. Method according to one of claims 1 to 13, characterized in that the nucleic acid is injected by the systemic route.

16. Method according to claim 15, characterized in that the nucleic acid is injected by the intra-arterial or intravenous route.

17. Method according to one of claims 1 to 13, characterized in that the nucleic acid is administered by the topical, cutaneous, oral, vaginal, intranasal, subcutaneous or intraocular route.

18. Method according to one of claims 1 to 17, characterized in that the nucleic acid is present in a composition containing, in addition, pharmaceutically acceptable excipients for the different modes of administration.

19. Composition according to claim 18, suitable for parenteral administration.

20. Method according to one of claims 1 to 19, characterized in that the nucleic acid is a deoxyribonucleic acid.

21. Method according to one of claims 1 to 19, characterized in that the nucleic acid is a ribonucleic acid.

22. Method according to one of claims 1 to 21, characterized in that the nucleic acid is of synthetic or biosynthetic origin, or extracted from a virus or from a unicellular or pluricellular eukaryotic or prokaryotic organism.

23. Method according to claim 22, characterized in that the nucleic acid administered is combined with all or part of the components of the organism of origin and/or of the synthesis system.

24. Method according to one of claims 1 to 23, characterized in that the nucleic acid encodes an RNA or a protein of interest.

25. Method according to claim 24, characterized in that the RNA is a catalytic or antisense RNA.

26. Method according to claim 24, characterized in that the nucleic acid encodes a protein chosen from enzymes, blood derivatives, hormones, lymphokines, growth factors, trophic factors, angiogenic factors, neurotrophic factors, bone growth factors, haematopoietic factors, coagulation factors, antigens and proteins involved in the metabolism of amino acids, lipids and other essential constituents of the cell.

27. Method according to claim 26, characterized in that the nucleic acid encodes the angiogenic factors VEGF and FGF, the neurotrophic factors BDNF, CNTF, NGF, IGF, GMF, aFGF, NT3, NT5, the Gax protein, insulin for the treatment of diabetes, growth hormone, calcitonin, leptin and the apolipoproteins, the enzymes for the biosynthesis of vitamins, hormones and neuromediators.

28. Method according to claim 24, characterized in that the nucleic acid codes for an antibody, a variable fragment of single-chain antibody (ScFv) or any other antibody fragment possessing recognition capacities for the purposes of immunotherapy, or codes for a soluble receptor, a peptide which is an agonist or antagonist of a receptor or of an adhesion protein, for an artificial, chimeric or truncated protein.

29. Method according to claim 28, characterized in that the nucleic acid encodes an antiidiotype antibody, a soluble fragment of the CD4 receptor or of the TNFα receptor or of the acetylcholine receptor.

30. Method according to one of claims 26 to 29, characterized in that the nucleic acid encodes a precursor of a therapeutic protein.

31. Method according to one of claims 1 to 30, characterized in that the nucleic acid is in the form of a plasmid.

32. Method according to one of claims 1 to 30, characterized in that the nucleic acid contains a gene of large size and/or introns and/or regulatory elements of small or large size.

33. Method according to one of claims 1 to 30, characterized in that the nucleic acid is an episomal DNA or a yeast artificial chromosome or a minichromosome.

34. Method according to one of claims 1 to 33, characterized in that the nucleic acid contains sequences allowing and/or promoting the expression of the transgene in the muscle.

35. Method according to one of claims 1 to 34, characterized in that the acid is combined with any type of vectors or with any combination of vectors which make it possible to improve the transfer of nucleic acid, such as viruses, synthetic or biosynthetic agents, or beads which are propelled or otherwise.

36. Method according to one of claims 1 to 35, characterized in that the muscle is subjected to a treatment intended to improve gene transfer, a treatment of pharmacological nature in the form of a local or systemic application, or an enzymatic, permeabilizing, surgical, mechanical, thermal or physical treatment.

37. Method according to one of claims 1 to 36, characterized in that it makes it possible to cause the muscle to produce an agent at physiological and/or therapeutic doses, either in the muscle cells, or secreted.

38. Method according to one of claims 1 to 36, characterized in that it makes it possible to modulate the quantity of transgene expressed by modulating the volume of muscle tissue transfected.

39. Method according to claim 38, characterized in that it makes it possible to modulate the volume of muscle tissue transfected by the use of multiple sites of administration.

40. Method according to one of claims 1 to 39, characterized in that it makes it possible to modulate the quantity of transgene expressed by modulating the number, shape, surface and arrangement of the electrodes, and by varying the intensity, the number, the duration, the frequency and the form of the pulses, as well as the quantity and the volume of nucleic acid for administration.

41. Method according to one of claims 1 to 40, characterized in that it makes it possible to control the localization of the tissues transfected by the volume of tissue subjected to the local electrical pulses.

42. Method according to one of claims 1 to 41, characterized in that it allows a return to the initial situation by removal of the transfected tissue area.

43. Nucleic acid and electric field of an intensity between 1 and 800 volts/cm, as combination product for their administration simultaneously, separately or spaced out over time in vivo to the striated muscle and, for gene therapy based on in vivo electrotransfection into the striated muscle.

44. Combination product according to claim 43, characterized in that the field intensity is between 4 and 400 volts/cm.

45. Combination product according to claim 43, characterized in that the field intensity is between 30 and 300 volts/cm.

46. Combination product according to one of claims 43 to 45, characterized in that the total duration of application of the electric field is greater than 10 milliseconds.

47. Combination product according to one of claims 43 to 46, characterized in that the application, to the muscle, of the electric field comprises one or more pulses of regular frequency.

48. Combination product according to claim 47, characterized in that the application, to the muscle, of the electric field comprises between 1 and 100,000 pulses of frequency between 0.1 and 10,000 hertz.

49. Combination product according to one of claims 43 to 46, characterized in that the electrical pulses are delivered in an irregular manner relative to each other and in that the function describing the intensity of the electric field as a function of the time for one pulse is variable.

50. Combination product according to one of claims 43 to 49, characterized in that the integral of the function describing the variation of the electric field with time is greater than 1 kVxmsec/cm.

51. Combination product according to claim 50, characterized in that this integral is greater than or equal to 5 kVxmsec/cm.

52. Combination product according to one of claims 43 to 51, characterized in that the electrical pulses are chosen from square wave pulses, electric fields generating exponentially decreasing waves, oscillating unipolar waves of limited duration, oscillating bipolar waves of limited duration, or other wave forms.

53. Combination product according to one of claims 43 to 52, characterized in that the electrical pulses comprise square wave pulses.

54. Combination product according to one of claims 43 to 53, characterized in that the electrical pulses are applied in an external manner.

55. Combination product according to one of claims 43 to 53, characterized in that the electrical pulses are applied inside the muscle.

56. Combination product according to one of claims 43 to 55, characterized in that the nucleic acid is injected into the muscle.

57. Combination product according to one of claims 43 to 55, characterized in that the nucleic acid is injected by the systemic route.

58. Combination product according to claim 57, characterized in that the nucleic acid is injected by the intra-arterial or intravenous route.

59. Combination product according to one of claims 43 to 55, characterized in that the nucleic acid is administered by the topical, cutaneous, oral, vaginal, intranasal, subcutaneous or intraocular route.

60. Combination product according to one of claims 43 to 59, characterized in that the nucleic acid is present in a composition containing, in addition, pharmaceutically acceptable excipients for the different modes of administration.

61. Composition according to claim 60, suitable for parenteral administration.

62. Combination product according to one of claims 43 to 61, characterized in that the nucleic acid is a deoxyribonucleic acid.

63. Combination product according to one of claims 43 to 61, characterized in that the nucleic acid is a ribonucleic acid.

64. Combination product according to one of claims 43 to 63, characterized in that the nucleic acid is of synthetic or biosynthetic origin, or extracted from a virus or an unicellular or pluricellular eukaryotic or prokaryotic organism.

65. Combination product according to claim 64, characterized in that the nucleic acid administered is combined with all or part of the components of the organism of origin and/or of the synthesis system.

66. Combination product according to one of claims 43 to 65, characterized in that the nucleic acid encodes an RNA or a protein of interest.

67. Combination product according to claim 66, characterized in that the RNA is a catalytic or antisense RNA.

68. Combination product according to claim 66, characterized in that the nucleic acid encodes a protein chosen from enzymes, blood derivatives, hormones, lymphokines, cytokines, growth factors, trophic factors, angiogenic factors, neurotrophic factors, bone growth factors, haematopoietic factors, coagulation factors, antigens and proteins involved in the metabolism of amino acids, lipids and other essential constituents of the cell.

69. Combination product according to claim 68, characterized in that the nucleic acid encodes the angiogenic factors VEGF and FGF, the neurotrophic factors BDNF, CNTF, NGF, IGF, GMF, FGF1, NT3, NT5, the Gax protein, insulin for the treatment of diabetes, growth hormone, calcitonin, leptin and the apolipoproteins, the enzymes for the biosynthesis of vitamins, hormones and neuromediators.

70. Combination product according to claim 66, characterized in that the nucleic acid codes for an antibody, a variable fragment of single-chain antibody (ScFv) or any other antibody fragment possessing recognition capacities for the purposes of immunotherapy, or codes for a soluble receptor, a peptide which is an agonist or antagonist of a receptor or of an adhesion protein, for an artificial, chimeric or truncated protein.

71. Combination product according to claims 70, characterized in that the nucleic acid encodes an antiidiotype antibody, a soluble fragment of the CD4 receptor or of the TNFα receptor or of the acetylcholine receptor.

72. Combination product according to one of claims 68 to 71, characterized in that the nucleic acid encodes a precursor of a therapeutic protein.

73. Combination product according to one of claims 43 to 72, characterized in that the nucleic acid is in the form of a plasmid.

74. Combination product according to one of claims 43 to 72, characterized in that the nucleic acid contains a gene of large size and/or introns and/or regulatory elements of small or large size.

75. Combination product according to one of claims 43 to 72, characterized in that the nucleic acid is an episomal DNA or a yeast or bacterial artificial chromosome or a minichromosome.

76. Combination product according to one of claims 43 to 75, characterized in that the nucleic acid contains sequences allowing and/or promoting the expression of the transgene in the muscle.

77. Combination product according to one of claims 43 to 76, characterized in that the acid is combined with any type of vectors or with any combination of vectors which make it possible to improve the transfer of nucleic acid, such as viruses, synthetic or biosynthetic agents, or beads which are propelled or otherwise.

78. Combination product according to one of claims 43 to 77, characterized in that the muscle is subjected to a treatment intended to improve gene transfer, a treatment of pharmacological nature in the form of a local or systemic application, or an enzymatic, permeabilizing, surgical, mechanical, thermal or physical treatment.

79. Combination product according to one of claims 43 to 78, characterized in that it makes it possible to cause the muscle to produce an agent at physiological and/or therapeutic doses, either in the muscle cells, or secreted.

80. Combination product according to one of claims 43 to 78, characterized in that it makes it possible to modulate the quantity of transgene expressed by modulating the volume of muscle tissue transfected.

81. Combination product according to claim 80, characterized in that it makes it possible to modulate the volume of muscle tissue transfected by the use of multiple sites of administration.

82. Combination product according to one of claims 43 to 81, characterized in that it makes it possible to modulate the quantity of transgene expressed by modulating the number, shape, surface and arrangement of the electrodes, and by varying the intensity, the number, the duration, the frequency and the form of the pulses, as well as the quantity and the volume of nucleic acid for administration.

83. Combination product according to one of claims 43 to 82, characterized in that it makes it possible to control the localization of the tissues transfected by the volume of tissue subjected to the local electrical pulses.

84. Combination product according to one of claims 43 to 83, characterized in that it allows a return to the initial situation by removal of the transfected tissue area.

5

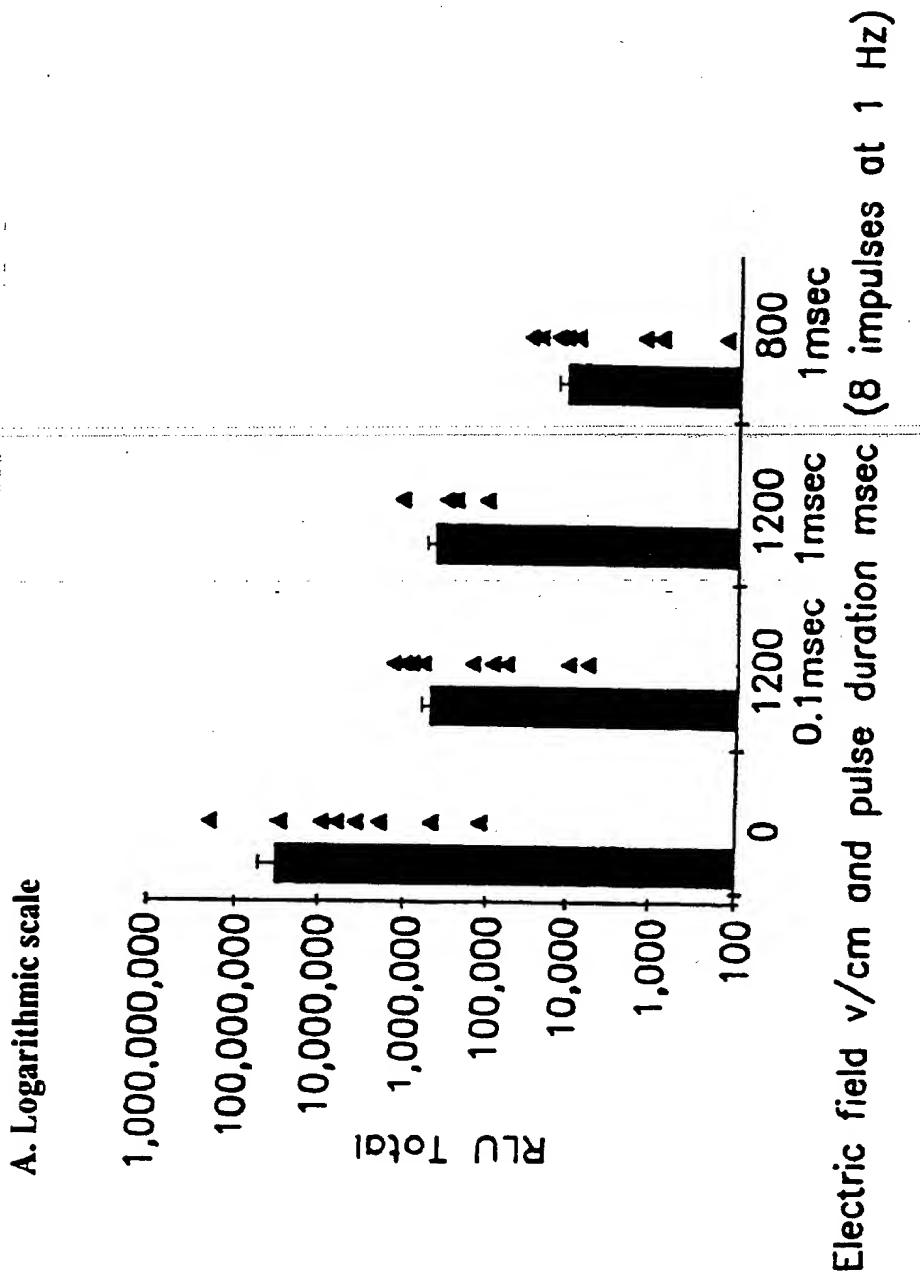

Figure 1a

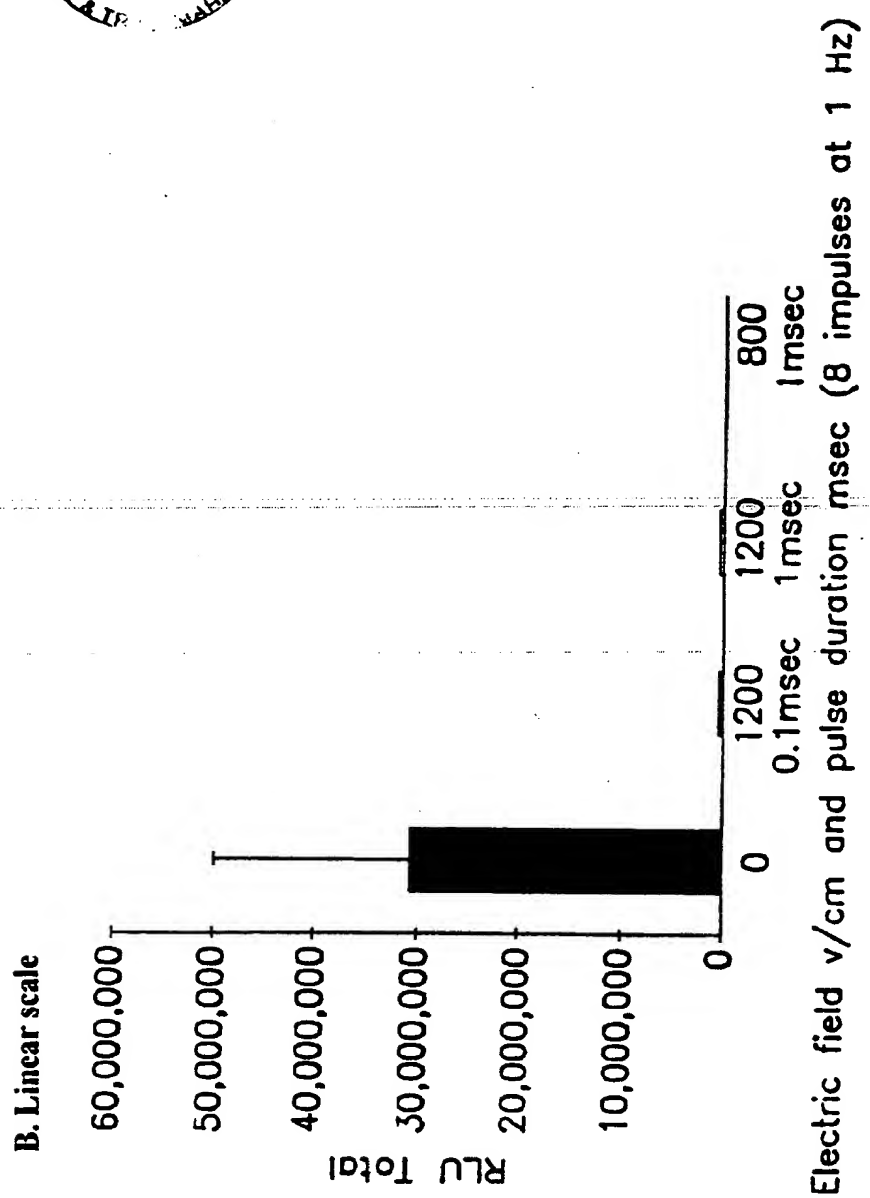

Figure 1b

A. Logarithmic scale

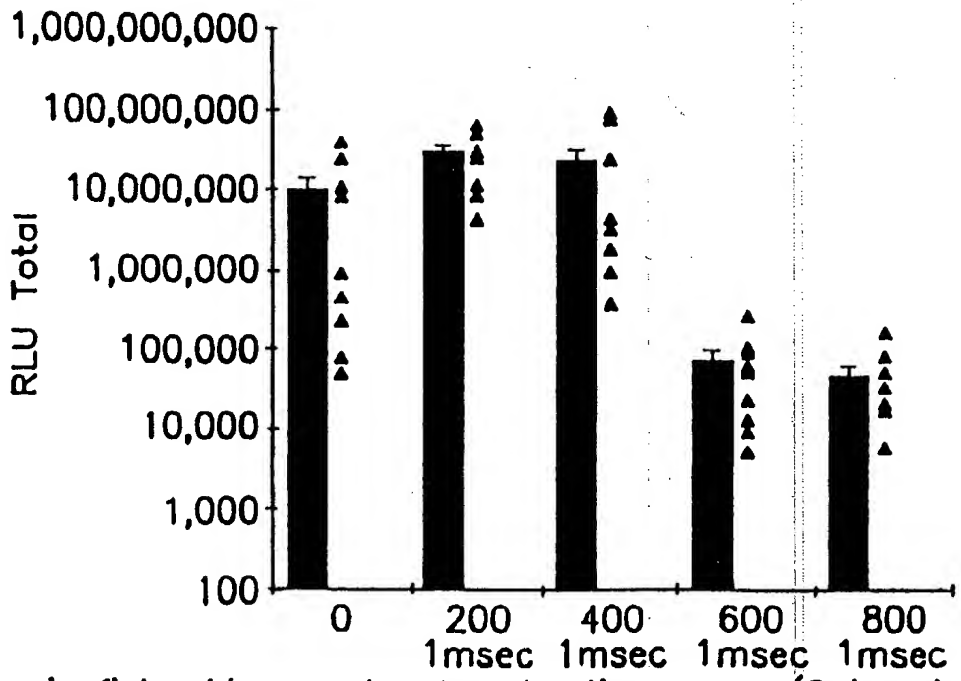

Figure 2a

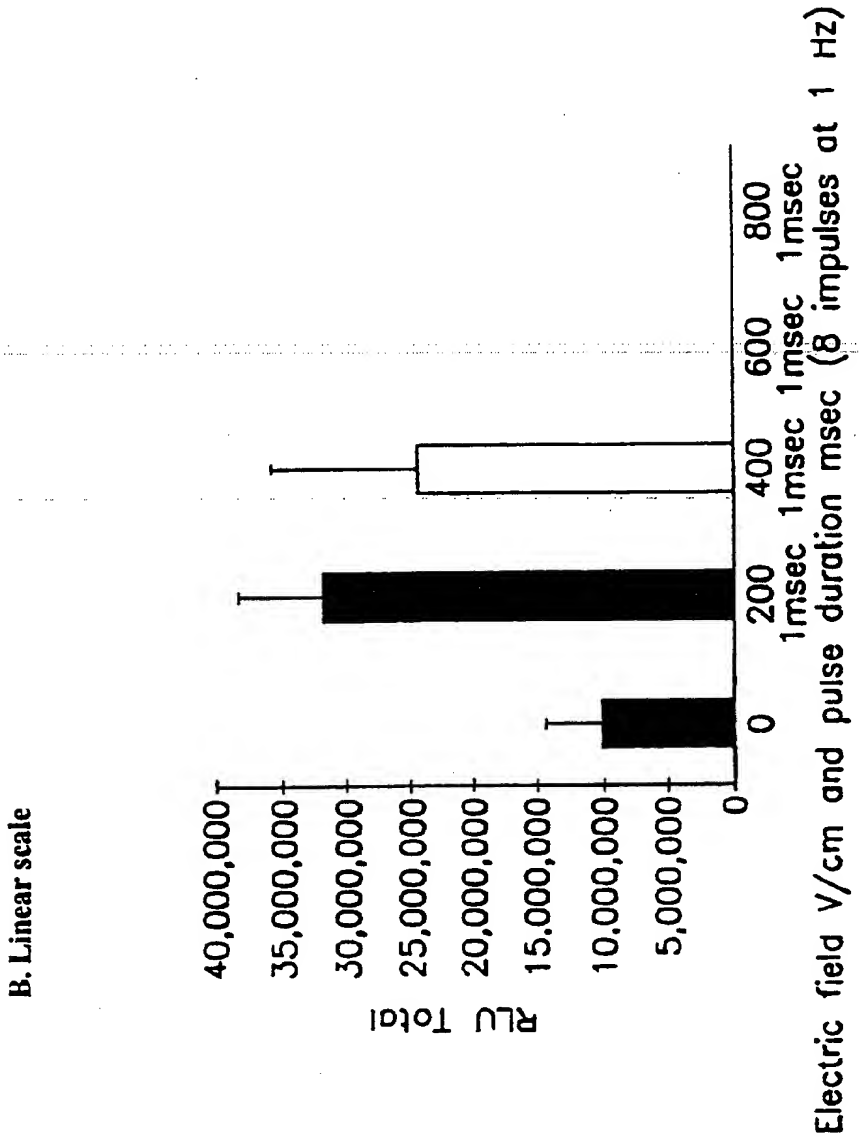

Figure 2b

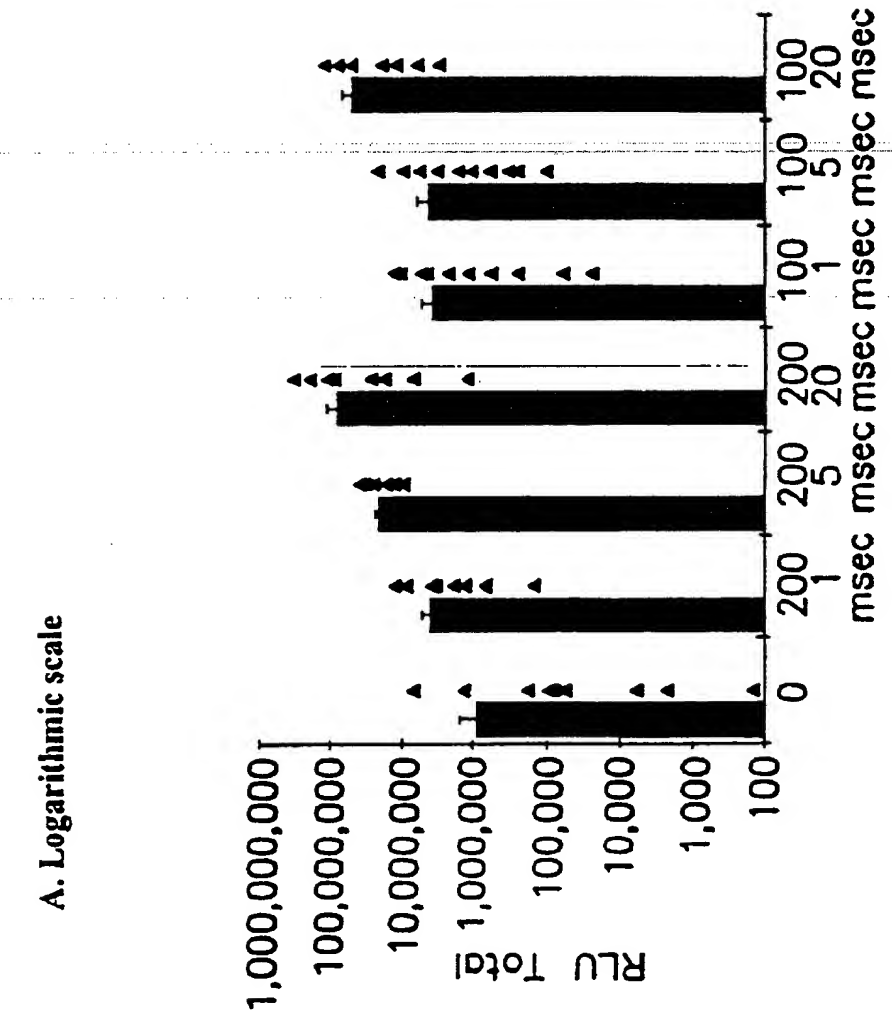

Figure 3a

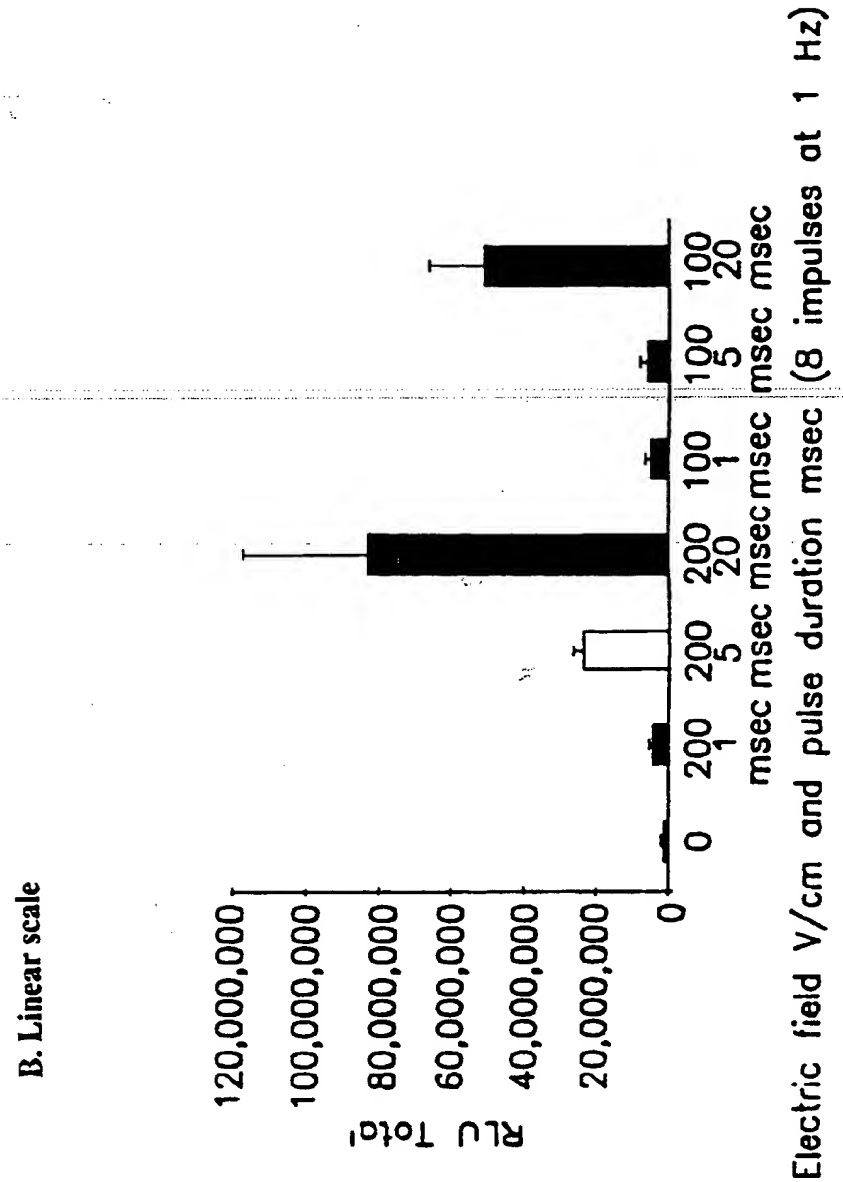

Figure 3b

A. Logarithmic scale

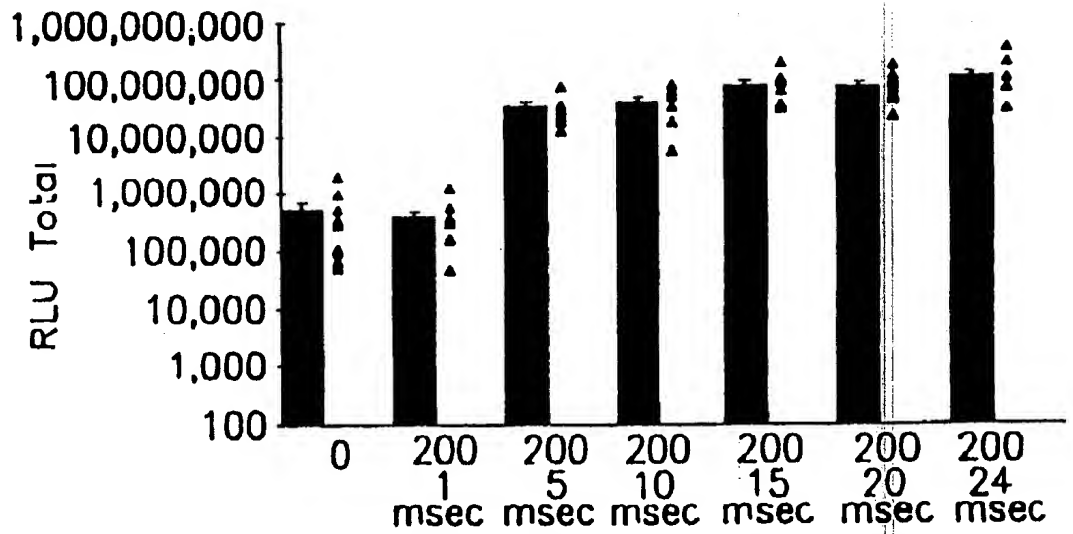

Figure 4a

B. Linear scale

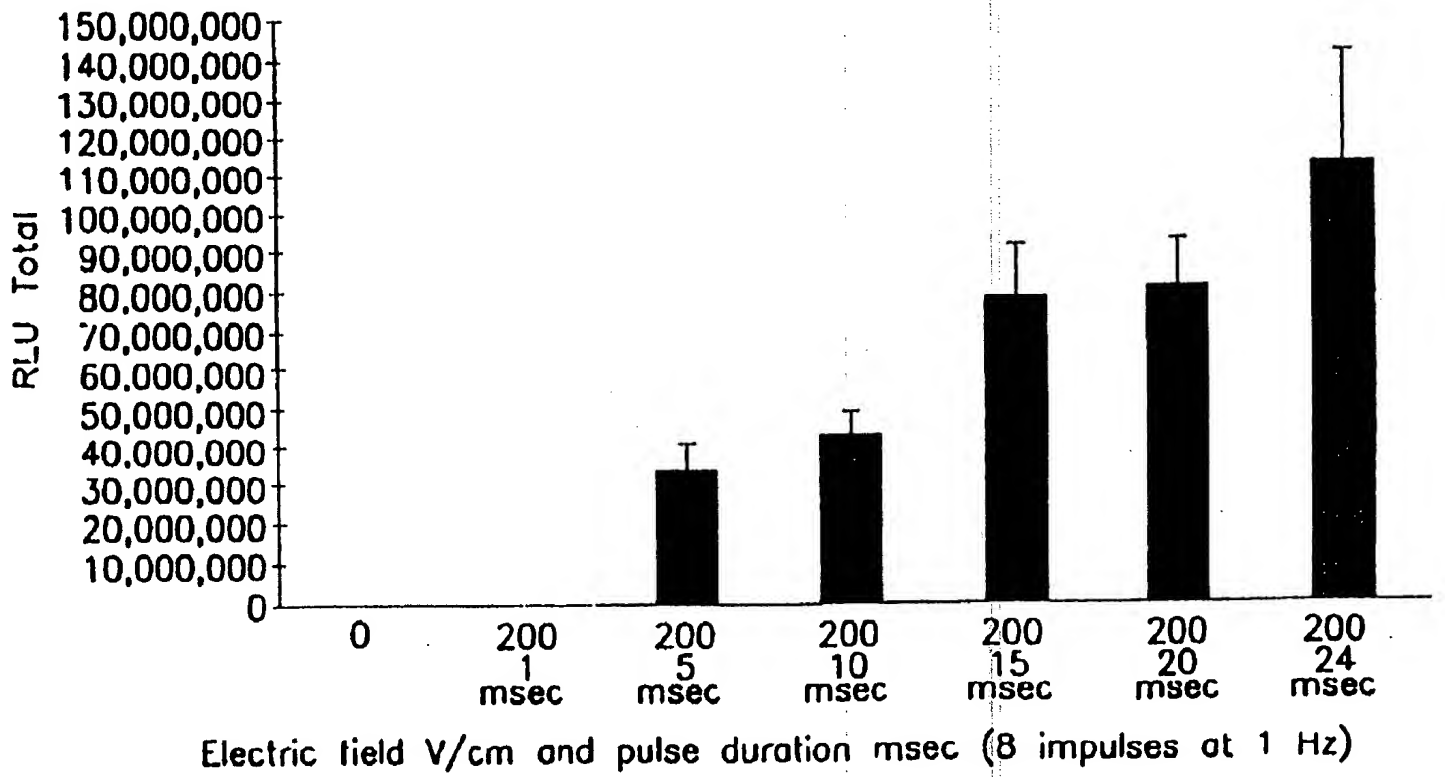

Figure 4b

Luciferase expression in relation to the mean value of the control group (DNA alone) which was standardized at 1.

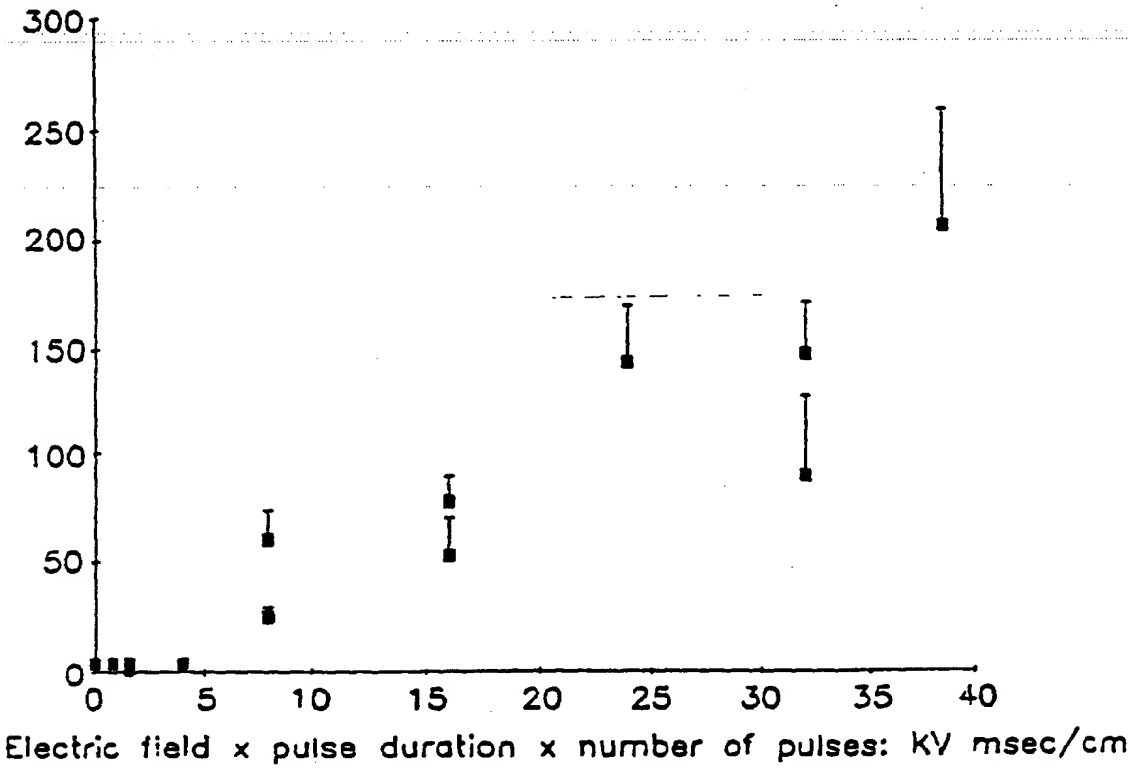

Figure 5